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Survey of the Distribution of a Newly Characterized Receptor for Advanced Glycation End Products in Tissues

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Advanced glycation end products (AGEs), the final products of nonenzymatic glycation and oxidation of proteins, are found in the plasma and accumulate in the tissues during aging and at an accelerated rate in diabetes. A novel integral membrane protein, termed receptor for AGE (RAGE), forms a central part of the cell surface binding site for AGEs. Using monospecific, polyclonal antibody raised to human recombinant and bovine RAGE, immunostaining of bovine tissues showed RAGE in the vasculature, endothelium, and smooth muscle cells and in mononuclear cells in the tissues. Consistent with these data, RAGE antigen and mRNA were identified in cultured bovine endothelium, vascular smooth muscle, and monocyte-derived macrophages. RAGE antigen was also visualized in bovine cardiac myocytes as well as in cultures of neonatal rat cardiac myocytes and in neural tissue where motor neurons, peripheral nerves, and a population of cortical neurons were positive. In situ ***hybridization confirmed the presence of RAGE mRNA in the tissues, and studies with rat PC12 pheochromocytes indicated that they provide a neuronal-related cell culture model for examining RAGE expression. Pathological studies of human atherosclerotic plaques showed infiltration of RAGE-expressing cells in the expanded intima. These results indicate that RAGE is present in multiple tissues and suggest the potential relevance of AGE-RAGE interactions for modulating properties of the vasculature as well as neural and cardiac function, prominent areas of involvement in diabetes and in the normal aging process. (Am J Pathol 1993, 143:1699–1712)***

The ultimate products of nonenzymatic glycation and oxidation of proteins by aldoses are termed advanced glycation end products (AGEs), an heterogeneous class of structures characterized by their yellow-brown color, fluorescence, propensity to form cross-links, and interaction with cellular receptors.[1–3] AGEs have been found in the plasma and tissues in aging, and their formation and deposition is enhanced in diabetes.[1–4] Their presence in the tissues and vessel wall is correlated with altered properties, such as decreased elasticity and trapping of plasma proteins, including low-density lipoprotein and IgG.[1]

AGEs may perturb cellular functions by interacting with receptors that we have recently characterized on cultured endothelial cells and mononuclear phagocytes.[5–7] The cell surface AGE binding site is comprised of an integral membrane protein, receptor for AGE (RAGE), and a noncovalently associated, lactoferrin-like polypeptide (LF-L), which binds to RAGE.[5] RAGE, a new member of the immunoglobulin superfamily, is comprised of an extracellular region with one V-type domain followed by two C-type domains. This receptor is highly conserved across the species; bovine and human RAGE are approximately 90% homologous.[6] Studies by Yang et al[8] have characterized two AGE binding proteins in rat liver whose reported amino terminal sequences are distinct from

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RAGE and LF-L. However, the relationship between these two sets of AGE binding proteins is unknown.

Using monospecific, polyclonal antibody raised to bovine RAGE and human recombinant RAGE, studies have been performed to identify RAGE in bovine tissues in homeostasis and in human atherosclerotic plaques. RAGE is found in endothelium, smooth muscle cells, cardiac myocytes, neural tissue, and mononuclear cells present in the tissues. Studies of RAGE antigen and mRNA indicate a wide distribution in different cell types. These experiments are a first step in the localization of RAGE in normal tissues and provide a foundation for more detailed future studies of human tissues in disease states.

Materials and Methods

Preparation and Characterization of Antibodies to RAGE

RAGE was purified from bovine lung as described[5] and was homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a single band with M_r approximately 30 to 35 kd (Figure 1A).[5] Purified RAGE was used to immunize rabbits by standard methods,[9] and IgG from immune serum was purified on protein A Sepharose (Pharmacia, Piscataway, NJ). The extracellular domain of human RAGE was expressed as a protein fusion in *Escherichia coli*. The open reading frame encoding amino acids Ala^1 through Ala^{293} of human RAGE was selectively amplified by polymerase chain reaction[10] using the oligomers 5′-TCTAGATCTCAAGCAGCACGGC TTTCCTGGGGCCCG 3′ and 5′-CTCAGATCTATC-GAAGGTCGCGCTCAAAACATC ACAGCCCGG 3′. These oligomeric sequences were incorporated into the RAGE reading frame appended to the factor Xa cleavage site Ile-Glu-Gly-Arg NH_2-terminal to Ala^1, a stop codon (TGA) to the COOH-terminal of Ala^{293} and *Bgl* II restriction enzyme sites at either ends of the DNA. The amplified fragment was digested with *Bgl* II, agarose gel isolated, and the purified fragment inserted into the *Bam*HI site of the T7 promoter expression vector pET3A.[11] Properly oriented clones were used to transform *E. coli* BL21 (DE3) cells[11] and transformants were grown in Luria Broth[12] supplemented with ampicillin (50 mg/L) until mid-log, when isopropyl-β-D-thiogalactoside (1 mM; Amersham, UK) was added for 3.5 hours. Cells were then harvested and sonicated and the insoluble, RAGE-containing fraction was processed as follows: solubilization in Tris/HCl (0.25 M; pH 8.0) containing guanidinium HCl (6 M), EDTA (10 mM), dithiothreitol (0.1 M), acidification to pH 3 with trifluoroacetic acid, centrifugation, and reversed-phase high performance liquid chromatography (POROS R/M) in trifluoroacetic acid (0.1%) with an ascending acetonitrile gradient (15 to 40%). Fractions with a single band, M_r approximately 31 kd, were pooled and NH_2-terminal sequencing demonstrated the ALA^2 of the T7 leader.

Purified recombinant human RAGE was used to immunize rabbits by standard method.[9] Control serum was both preimmune IgG from the same rabbits and pooled nonimmune rabbit IgG. Where indicated, anti-RAGE IgG (5 μg/ml) was incubated for 3 hours at 37°C with purified bovine RAGE (60 to 65 μg/ml), the mixture was centrifuged (10,000 × *g* for 30 minutes), and the supernatant used for immunohistochemical studies. Affinity-purified anti-RAGE IgG was prepared by adsorbing RAGE antiserum to

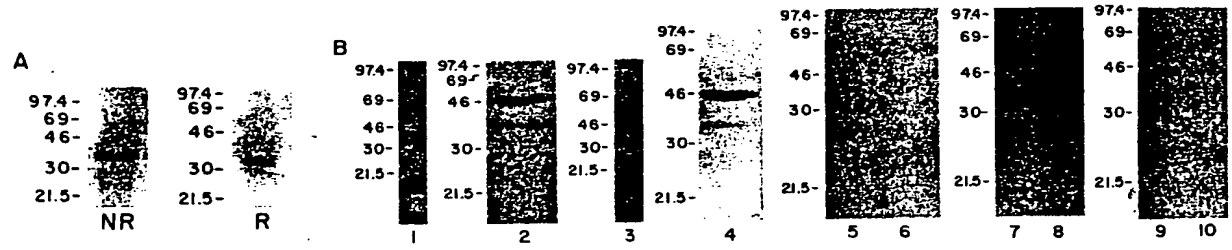


Figure 1. *Characterization of anti-bovine RAGE IgG and anti-human RAGE IgG by Western blotting of purified bovine RAGE and bovine lung extracts.* A: *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified bovine RAGE (1.0 μg/lane) on nonreduced (NR) and reduced (R) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) visualized by Coomassie blue staining.* B: *Bovine RAGE (1.5 μg/lane) or lung extract (195 μg/lane) was subjected to nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), electroblotting, and then blots were reacted with anti-bovine RAGE IgG (5 μg/ml), anti-human RAGE IgG (5 μg/ml), or nonimmune IgG (5 μg/ml): lane 1, anti-bovine RAGE IgG and immobilized purified RAGE; lane 2, anti-bovine RAGE IgG and immobilized lung extract; lanes 3 and 4, anti-human RAGE IgG and immobilized purified RAGE or lung extract, respectively; lanes 5 and 6, the experiment was performed as in lanes 1 and 2 except that soluble bovine RAGE (60 μg/ml) was added during incubation of anti-bovine RAGE IgG with the blots; lanes 7 and 8, the experiment was performed as in lanes 3 and 4, except that soluble bovine RAGE (60 μg/ml) was added during incubation of anti-human RAGE IgG with the blots; lanes 9 and 10, nonimmune IgG replaced immune IgG and immobilized purified RAGE or lung extract, respectively, was present on the blots. Migration of standard proteins is indicated.*

an affi-gel 10 column (BioRad, Hercules, CA) with immobilized RAGE (1 mg/ml resin), washing extensively with Tris-buffered saline (20 mM; pH 7.4) (NaCl, 0.1 M) containing Tween 20 (0.05%) and eluting bound immune IgG with acidic buffer (glycine, 0.2 M, pH 2.5). IgG was immediately neutralized and then dialyzed versus phosphate-buffered saline (PBS; pH 7.2).

Preparation and Immunocytochemistry of Tissues/Cultured Cells

Normal bovine tissues were obtained from the abattoir on ice, fixed overnight with formalin (3.5%) in PBS, dehydrated, and embedded in paraffin by standard procedures. Some tissue samples were placed in tissue freezing medium (Polysciences, Inc., Warrington, PA) and snap-frozen in dry ice/methanol for frozen sectioning. Samples of human tissue were obtained from the Department of Pathology (Columbia) and National Disease Research Institute (Bethesda, MD). For immunostaining of cultured cells, monolayers grown on coverslips were washed twice in Earle's balanced salt solution (37 C) and fixed in paraformaldehyde (3.5%) containing NP-40 (0.1%) in PBS.

Immunohistochemical studies used sections that were rehydrated, incubated in blocking buffer (bovine serum albumin [1%] and normal goat serum [2%] in PBS) for 30 minutes at 37 C, washed in PBS, exposed to primary antibody (20–40 μg/ml) for 45 minutes at 37 C, washed again, and incubated with biotinylated goat anti-rabbit immunoglobulin followed by peroxidase-conjugated avidin (Sigma, St. Louis, MO). Localization of peroxidase conjugates was revealed using diaminobenzidine tetrahydrochloride (DAB) or amino ethylcarbazole (AEC) as the chromogen.

Northern Blotting and In Situ *Hybridization*

Northern blots of human poly A^+ RNA (Clontech, Palo Alto, CA) were prepared from formaldehyde agarose gels (1.2%) that were transferred to Nytran nylon (Schleicher and Schuell, Keene, NH) and fixed by ultraviolet irradiation. The blot was hybridized in saline sodium phosphate ethylenediamine tetraacetic acid (SSPE) (5X), Denhardt's (10X), deionized formamide (50%), sodium dodecyl sulfate (2%), and salmon sperm DNA (100 μg/ml). The probe was the entire human RAGE cDNA ^{32}P-labeled by use of a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). After overnight hybridization at 42 C, the blot was washed in saline sodium citrate (SSC) (0.1X)/sodium dodecyl sulfate (0.1%) at 55 C and exposed to film. The blot was then washed in sterile water (70 C) and rehybridized with a human actin probe in the same manner as described for RAGE above. For certain experiments, hybridization was also performed at lower temperatures (55 C, 5X SSC, no formamide) and less stringent washes (55 C, 2X SSC) to look for the possible existence of related transcripts. For *in situ* hybridization, digoxigenin-labeled riboprobes were transcribed from the plasmid B379-2A[6] containing a 1406-bp fragment of bovine RAGE (from 1 to 1406 nt) cloned into the *Eco*RI site of pBluescript II SK. Anti-sense probe was transcribed from the T3 promoter with the plasmid linearized by *Xho* I. Control sense probe was transcribed from the T7 promoter with the plasmid linearized by *Xba* I. Transcription used an RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) with digoxigenin-UTP by *in vitro* transcription with either T3 or T7 polymerase. The digoxigenin-labeled RNA probe was hybridized to cellular mRNA and detected with anti-digoxigenin alkaline phosphatase-conjugated antibody. Antibody was visualized with X-phosphate and nitroblue tetrazolium salt.

Western Blot Analysis and ELISA of Bovine Tissues/Cultured Cells for RAGE Antigen

Western blot analysis was performed on samples of bovine tissue obtained immediately after sacrifice of the animals. Tissues were cut into approximately 1-mm pieces, homogenized in a Waring blender in acetone, and then exposed sequentially to chloroform followed by acetone (50 ml/10 g of tissue; three successive incubations for 24 hours at 4 C in each solvent) followed by drying. The homogenate was extracted in Tris (20 mM; pH 7.4), NaCl (100 mM), phenylmethylsulfonyl fluoride (1 mM), and octyl-β-glucoside (1%) overnight at 4 C (1.5 g of tissue was extracted with 5 ml of buffer) and centrifuged (11,000 × *g* for 30 minutes). Supernatants were subjected to nonreduced sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) followed by electroblotting onto nitrocellulose. The membranes were then incubated in nonfat dry milk as described.[13] Immunoreactive bands were identified by the horseradish peroxidase method using DAB. Molecular weights were interpolated from

semilogarithmic plots constructed based on the migration of standard proteins (Amersham, Arlington Heights, IL): myosin, 200 kd; phosphorylase b, 97.4 kd; bovine serum albumin, 69 kd; ovalbumin, 46 kd; carbonic anhydrase, 30 kd; and trypsin inhibitor, 21.5 kd. ELISA for RAGE was performed using purified anti-human RAGE antibody as follows.

Tissues and cells were extracted as above, and protein concentrations determined using the Bio-Rad protein assay kit according to the manufacturer's instructions. Equivalent amounts (based on protein content) of each extract were incubated in wells (0.1 ml) of Nunc Maxi-Sorp plates (Napersville, IL) overnight at 4 C. Wells were then washed with PBS containing Tween 20 (0.05%) and blocked with PBS containing goat serum (5%; 0.1 ml) for 2 hours at 37 C. Wells were washed and incubated with anti-RAGE IgG (30 μg/ml; 0.1 ml) in PBS containing goat serum (1%) for 2 hours at 37 C. Wells were again washed, incubated for 1 hour at 37 C with peroxidase-conjugated goat anti-rabbit IgG (1:3000 dilution; 0.1 ml) then washed and developed with *O*-phenylenediamine dihydrochloride (OPD) according to the manufacturer's instructions (Sigma) for approximately 10 minutes. The reaction was stopped with H_2SO_4 (2 M) and adsorbance at 490 nm was determined. RAGE equivalent values (pg/ml) were determined using a standard curve prepared with human RAGE and the limit of detection in this assay was <50 pg/ml. Similar results were obtained with anti-bovine RAGE IgG and affinity-purified anti-bovine RAGE IgG.

Preparation of AGE Albumin-Impregnated Polytetrafluoroethylene (PTFE) Mesh for Subcutaneous implantation

Tubes of PTFE mesh (diameter 1 cm, length 2 cm, 90 μ pore size, Gortex; generously provided by W. L. Gore, Inc., Wakefield, MA) were incubated in solutions containing either AGE albumin (rat serum albumin [Sigma] incubated for 4 weeks in the presence of glucose [0.5 M] at 37 C, prepared as described)[7] or native albumin (native albumin had been incubated 4 weeks at 37 C in the same buffer but without glucose), each at a concentration of 500 μg/ml for 16 hours at 4 C. Based on studies with ^{125}I-AGE albumin and ^{125}I-albumin, adsorption of each of these proteins to the mesh was comparable and dissociation was slow, with greater than 70% of the initial material still adherent to the mesh after 7 days.[7] PTFE tubes were washed in saline and implanted into the subcutaneous tissue of rats for 5 days. At that time, the dermal layer containing the PTFE tubes was excised, fixed, sectioned, and stained as above.

Cell Culture

Bovine adrenal capillary endothelial cells were isolated as described previously.[14] Bovine vascular smooth muscle cells were prepared from bovine aortae after harvesting of endothelial cells and were characterized based on staining for smooth muscle actin.[15] Rat neonatal cardiac myocytes were prepared from cardiac tissue of newborn rats using a previously described method.[16] Cardiac myocytes were used in experiments 6 days after plating, at which time they were observed to be beating synchronously. The PC12 line (provided by Dr. L. Greene, Dept. of Pathology), derived from a transplantable adrenal medullary pheochromocytoma, was grown in culture and exposed to nerve growth factor (NGF; 10 nM) for 48 hours, as described.[17] Cultured mesangial cells[18] were generously provided by Dr. J. Oliver (Dept. of Medicine).

Results

Characterization of Polyclonal anti-RAGE IgG

RAGE purified from bovine lung migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with M_r approximately 30 to 35 kd, similarly under nonreduced and reduced conditions (Figure 1A). IgG from immune rabbit serum raised against purified bovine RAGE or recombinant human RAGE was reacted with Western blots containing purified bovine RAGE or lung extract (Figure 1B, lanes 1 to 2, anti-bovine RAGE IgG; lanes 3 to 4, anti-human RAGE IgG). A single band, M_r approximately 30 to 35 kd, was observed with purified RAGE (lane 1); the latter was not observed when excess purified RAGE was added during incubation of primary antibody with the blots (Figure 1B, lane 5, anti-bovine RAGE IgG; lane 7, anti-human RAGE). Immunoblotting of unconcentrated crude lung extract showed bands at approximately 35 kd and 46 kd (Figure 1B, lanes 2 and 4), whose appearance was also prevented when excess purified RAGE was present (Figure 1B, lanes 6 and 8). Neither nonimmune IgG (Figure 1B, lanes 9, purified RAGE; lane 10, lung extract)

nor preimmune IgG (data not shown) revealed any bands when immunoblots were processed in an identical manner.

Immunostaining studies also showed that anti-bovine RAGE IgG and anti-human RAGE IgG were comparable reagents for detection of RAGE in tissues (Figure 2). In sections of bovine lung, RAGE antigen was visualized similarly with each of these reagents, especially evident in the vessel wall of small arteries, venules, and alveolar capillaries (Figure 2A and B). Preincubation of either antibody with purified bovine RAGE prevented visualization of RAGE antigen (Figure 2C and D anti-bovine RAGE IgG and anti-human RAGE IgG, respectively). Nonimmune and preimmune serum revealed no staining (Figure 2E and F, respectively). These data indicate that the immunological reagents used in this study visualized RAGE antigen specifically.

Immunostaining of RAGE in Bovine Tissues

Using these immunological reagents to detect RAGE, a tissue survey was performed to identify the major cell types that demonstrated RAGE.

In the lung, in addition to the presence of RAGE in pulmonary endothelium (Figure 2), RAGE antigen was demonstrable in bronchial and vascular smooth muscle, as well as in alveolar macrophages and leiomyocytes (Figures 2B and 3A and B) and on the visceral pleural surface (data not shown). The presence of RAGE in smooth muscle led us to

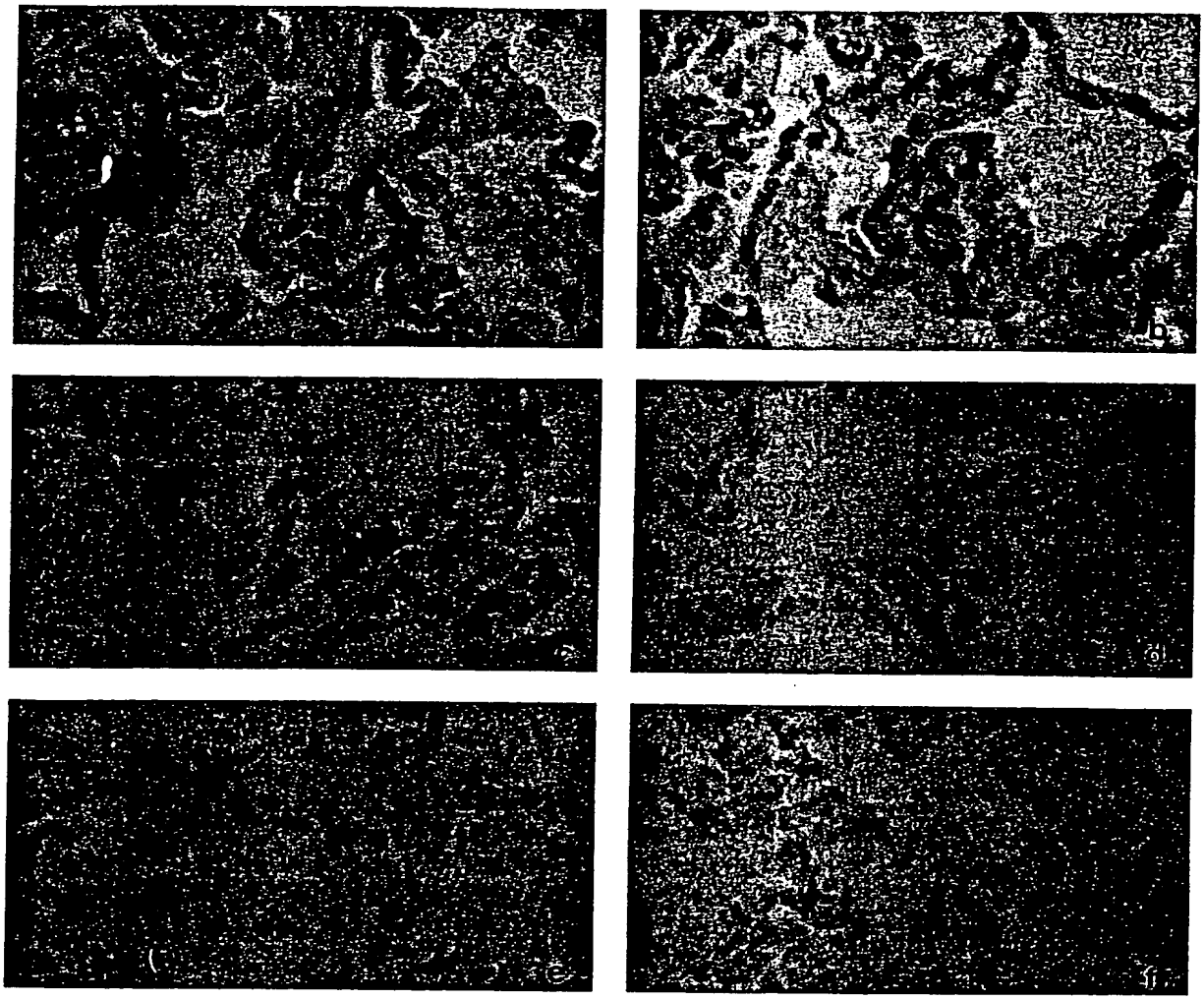


Figure 2. *Immunostaining of bovine lung tissue with either anti-bovine RAGE IgG (30 μg/ml)* (a *and* c), *anti-human RAGE IgG (30 μg/ml)* (b *and* d), *nonimmune IgG (30 μg/ml)* (e), *or preimmune IgG (30 μg/ml)* (f). *In* c *and* d *the anti-RAGE IgG (30 μg/ml) was preincubated with purified RAGE (60 μg/ml), centrifuged, and the supernatant applied to the sections. Samples were stained as described in the text using DAB* (a, c, *and* e) *or AEC* (b, d, *and* f) *as the chromogen. Magnification ×300.*

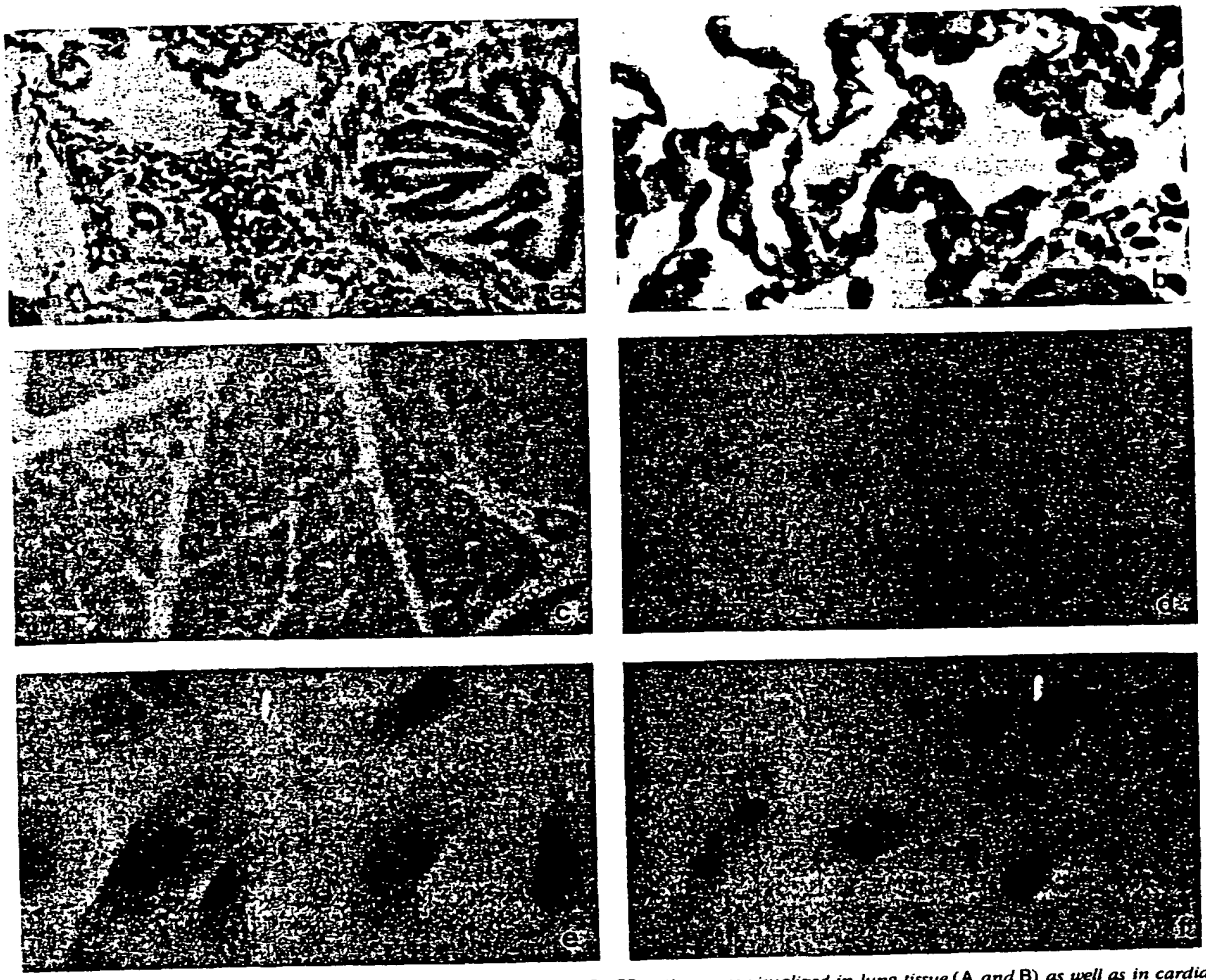


Figure 3. *Immunohistological detection of RAGE in lung and heart. RAGE antigen was visualized in lung tissue* (A *and* B) *as well as in cardiac myocytes* (C). D *represents a section of the same cardiac tissue stained with nonimmune IgG. Cultured cardiac myocytes also stained with anti-RAGE IgG* (E, *although no staining was observed with nonimmune IgG* (F) *Magnification:* A ×*120;* B ×*300;* C *and* D ×*130;* E *and* F ×*340.*

assess its expression in other types of musculature. In the heart, cardiac myocytes exhibited diffuse sarcoplasmic staining for RAGE (Figure 3C), whereas no staining was observed with nonimmune IgG (Figure 3D). Consistent with the demonstration of RAGE in cardiac muscle, RAGE was observed in cultured rat cardiac myocytes (Figure 3E), whereas staining of the same cells with nonimmune IgG was negative (Figure 3F).

In the vasculature, RAGE was most evident in the medial smooth muscle and in most, but not all, endothelial cells. Smooth muscle staining was seen in the media of a pulmonary arterial vessel and the aorta (Figure 4A and B, respectively). Endothelial cells and some pericytes of the microvasculature stained positively for RAGE antigen (Figure 4C), although staining of the microvasculature was not uniform among the capillary beds of different organs. Consistent with the presence of RAGE in these vessel wall-derived cells, cultures of both vascular smooth muscle and adrenal capillary endothelial cells were positive for RAGE antigen (Figure 4D and E, respectively), whereas reaction of these cells with nonimmune IgG showed no staining (Figure 4F and G).

The capillary bed of the normal kidney, as in the renal glomeruli, was consistently negative for immunohistochemically demonstrable RAGE, and the mesangial cells were weakly reactive (Figure 5A). Medial smooth muscle and endothelium of afferent arterioles stained for RAGE (Figure 5A), although the efferent arteriole and cells of the juxtaglomerular apparatus were unstained. Studies with cultured mesangial cells also indicated that RAGE was present (Figure 5C, anti-RAGE IgG and D, nonimmune IgG). Of the epithelial components of the kidney, only cells of the proximal convoluted tubule and occasionally

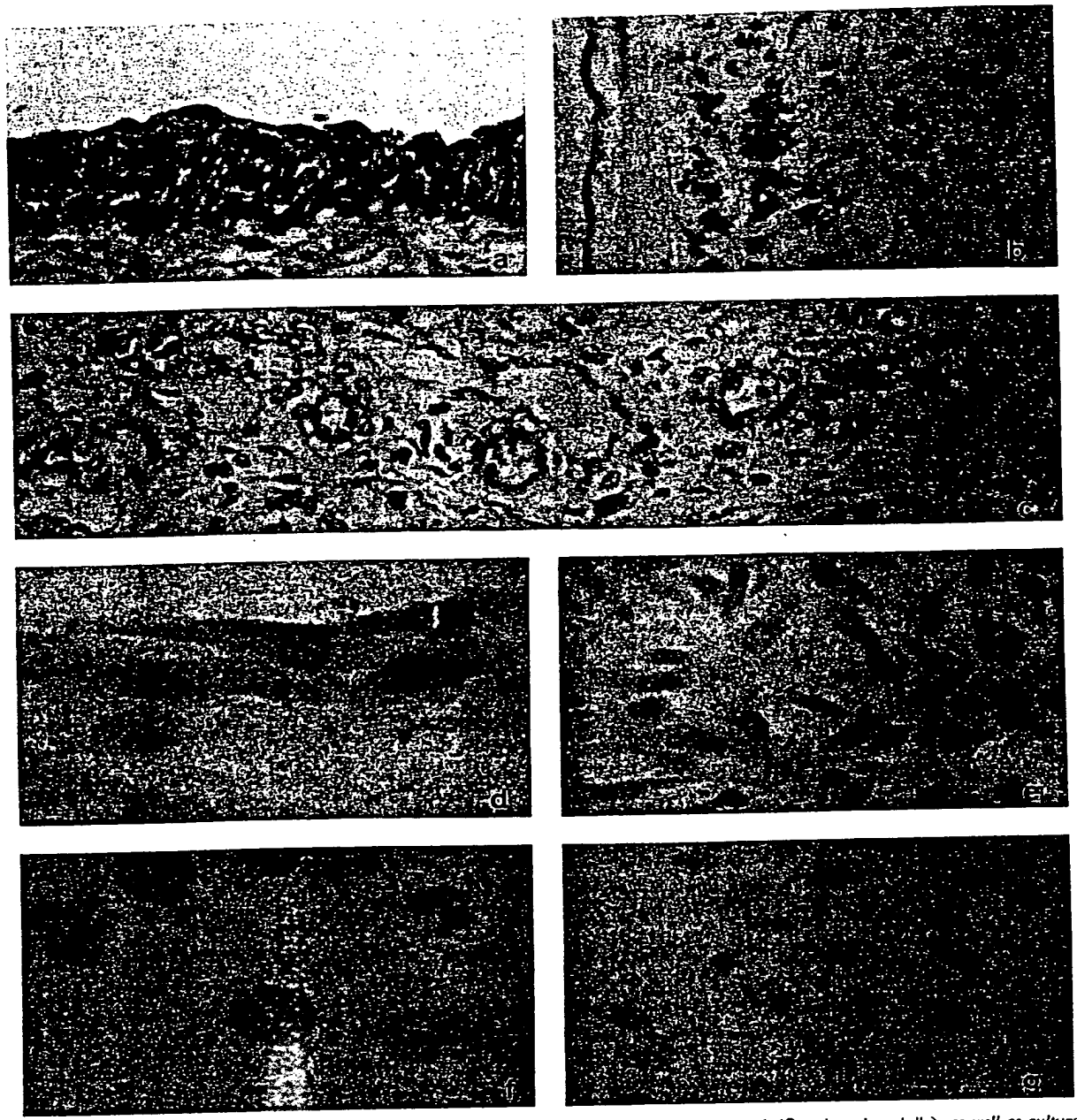


Figure 4. *Detection of RAGE in arterial vessels* (A, *pulmonary artery and* B, *aorta*) *and microvessels* (C, *adrenal medulla*), *as well as cultured vascular smooth muscle* (D, *anti-RAGE IgG and F, nonimmune IgG*) *and adrenal microvascular endothelium* (E, *anti-RAGE IgG and F, nonimmune IgG*). *Magnification:* A *to* F ×340.

those of the parietal layer of Bowman's capsule contained RAGE antigen; those of the distal nephron were consistently unstained (Figure 5B). Within cells of the proximal tubules, RAGE antigen was visualized only basolaterally, suggesting that this material might represent readsorption of RAGE present in the plasma after cleavage from the cell surface, a possibility that is currently being explored.

In the hepatocyte (Figure 5E), staining was concentrated in a juxtanuclear focus in addition to faint staining elsewhere in the cytoplasm. In contrast, epithelia of the alimentary tract were consistently negative in sections that stained strongly for RAGE in muscular layers, neurons of the submucosa, and most myenteric plexi, as well as macrophages within the propria in the colon (Figure 5F).

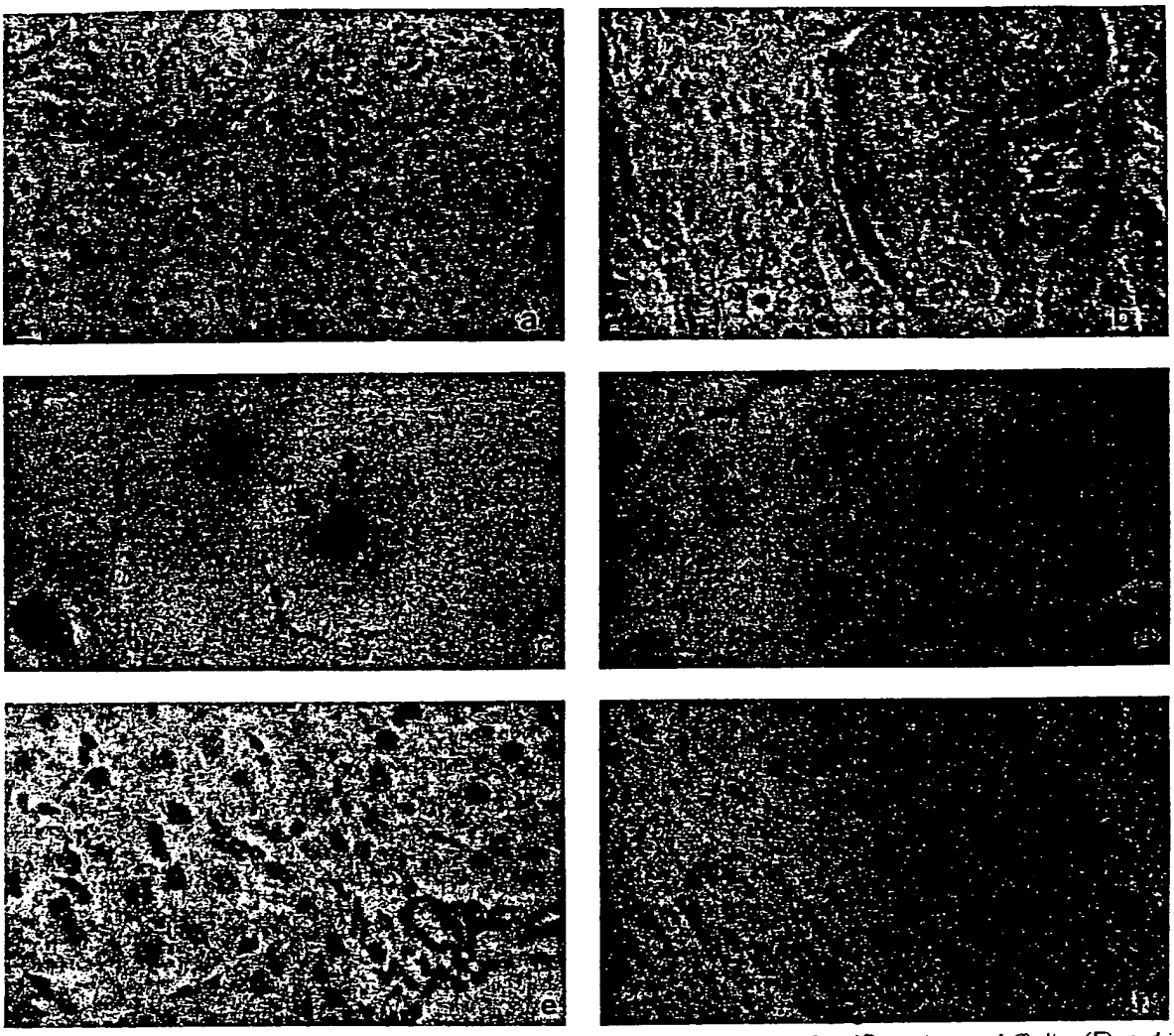


Figure 5. *Immunostaining of bovine kidney* (A *and* B), *cultured mesangial cells* (C, *anti-RAGE IgG and* D, *nonimmune IgG*), *liver* (E), *and intestine* (F) *for RAGE. Magnification:* A ×*200*; B ×*300*; C *and* D ×*300*; *E* ×*340*; F ×*120*.

Within the central nervous system, RAGE was localized to particular sets of neurons and in the ependyma and microvasculature but not in normal resting glia (Figure 6B). Spinal motor neurons of the anterior horn were stained, especially Nissl bodies (Figure 6A). The presence of RAGE in neural tissue led us to examine expression of the receptor in cultured PC12 pheochromocytes. Unstimulated PC12 cells demonstrated a diffuse pattern of surface staining, although normal cells of resting adrenal medulla as well as other quiescent neuroendocrine cells *in situ* were immunohistologically negative (data not shown). After treatment of PC12 cells with NGF, RAGE was distributed in the neurite outgrowths as well as in the cell bodies (Figure 6C).

Although circulating monocytes, resting histiocytes, and Kupffer cells did not contain RAGE, activated derivatives of monocytic lineage, including alveolar macrophages and colonic macrophages were positive. In addition, macrophages infiltrating AGE-impregnated polytetrafluoroethylene mesh and giant cells (Figure 7A), as well as foam cells and proliferating smooth muscle cells of early atheroma, were strongly positive for RAGE (Figure 7B).

Immunoblotting and Detection of RAGE mRNA

Immunoblotting, performed to detect forms of RAGE present in the tissues (Figure 8A), demon-

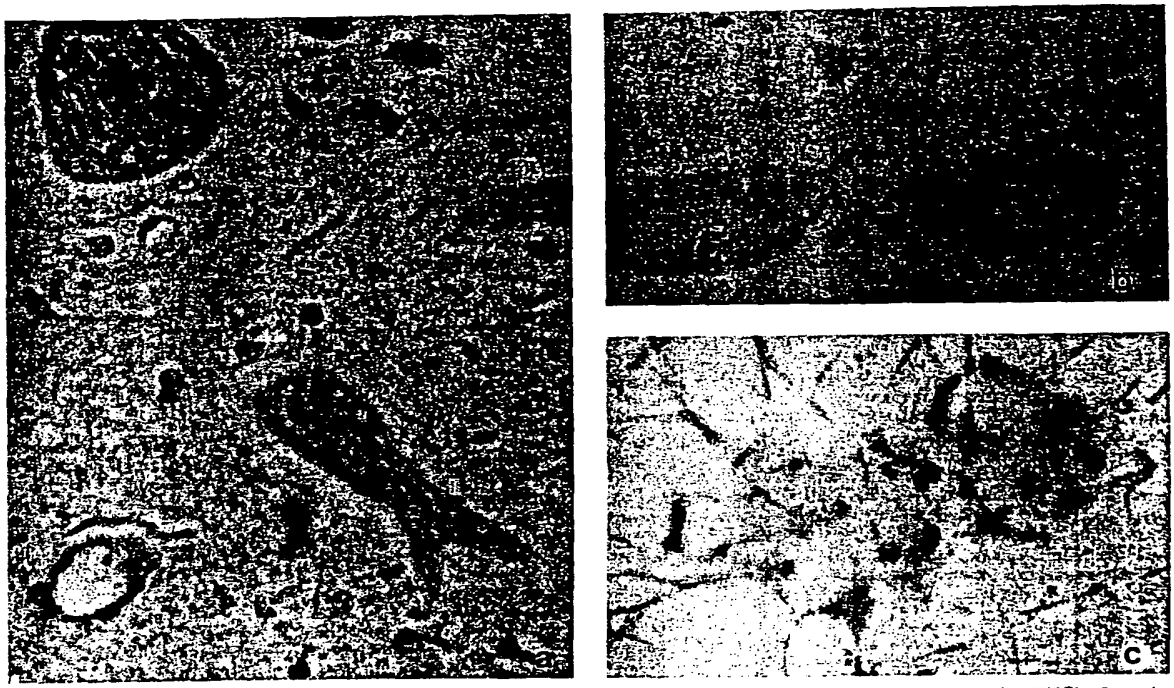


Figure 6. *Immunocbemical detection of RAGE in spinal motor neurons* (A), *cerebral cortex* (B), *and PC12 cells treated with NGF* (C). *Control experiments with PC12 cells using nonimmune IgG in place of anti-RAGE IgG were negative* (*data not shown*). *Magnification:* A ×*340*; B ×*140*; C ×*700*.

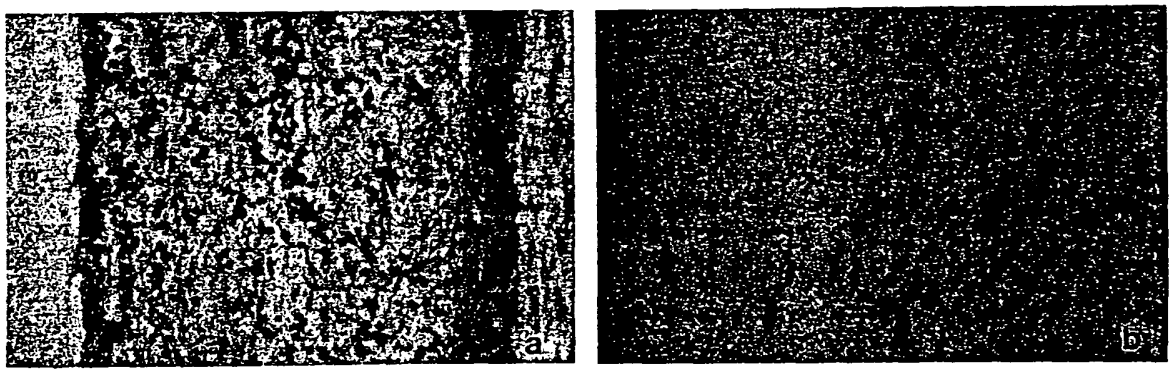


Figure 7. A: *RAGE was demonstrable in activated mononuclear cells infiltrating PTFE mesh impregnated with AGE albumin implanted for 5 days in the subcutaneous tissue of a rat. Foreign body giant cells at the border of the implant with the connective tissue also stained intensely.* B: *In the early stage of development of an atheromatous lesion in the intima of a human coronary artery from a 20-year-old male, infiltrating cells were immunostained for RAGE, as was the overlying endothelium. Magnification:* ×*300*.

strated two major bands with M_rs between 35 and 45 kd (lanes 1 to 7), whose appearance was blocked when excess purified RAGE was added during incubation of blots with the primary antibody (lanes 8 to 14). In the case of the brain, however, the M_rs of the immunoreactive material were slightly different (Figure 8A, lane 4; M_rs of approximately 23 and 48 kd). Both of these bands that were immunoreactive with anti-RAGE IgG also disappeared in the presence of excess purified RAGE (lane 11). Similar results were obtained both with anti-bovine RAGE IgG and anti-human RAGE IgG. ELISA of the different tissue extracts using anti-RAGE IgG demonstrated the antigen to be most abundant in the heart, lung, and skeletal muscle (Figure 8B), which approximated the results observed in the immunohistochemical and Western blotting studies. Consistent with the expression of RAGE in a spectrum of organs, ELISA of extracts from cultured endothelial cells (ECs), vascular smooth muscle cells, mesan-

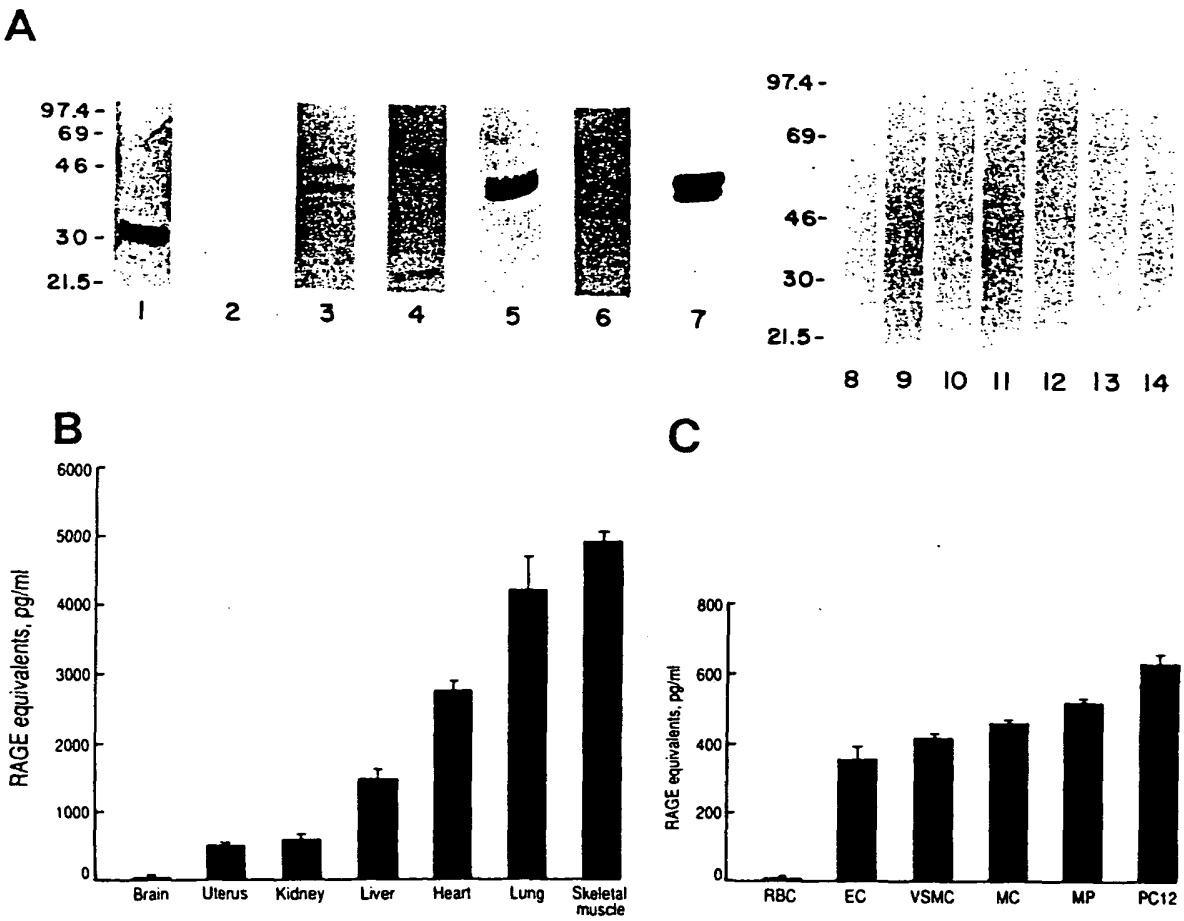


Figure 8. *Western blotting and ELISA for RAGE in bovine tissue extracts/cultured cells.* A: *Tissues were harvested and extracts were prepared subjected to nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and immunoblotted with anti-human RAGE IgG (5 μg/ml) (lanes 1 to 7) or anti-human RAGE IgG (5 μg/ml) in the presence of soluble RAGE (65 μg/ml) (lanes 8 to 14). Samples consisted of purified bovine RAGE (lanes 1 and 8, 1.5 μg/lane) and extracts of uterus (lanes 2 and 9, 340 μg/lane), heart (lanes 3 and 10, 21 μg/lane), brain (lanes 4 and 11, 350 μg/lane), liver (lanes 5 and 12, 21 μg/lane), kidney (lanes 6 and 13, 300 μg/lane), and skeletal muscle (lanes 7 and 14; 14 μg/lane). The migration of standard proteins run simultaneously is indicated in kd. Approximate M_rs of immunoreactive bands: lane 1 (purified RAGE), 30 kd; lane 2 (uterus), 32 and 46 kd; lane 3 (heart), 38 and 42 kd; lane 4 (brain), 23 and 48 kd; lane 5 (liver), 40 kd; lane 6 (kidney), 36 and 40 kd; and lane 7 (skeletal muscle), 40 and 42 kd.* B: *Tissue extracts were prepared, and equivalent amounts based on protein content were analyzed for RAGE content by ELISA as described in the text. RAGE equivalent values (mean ± SE in pg/ml) were calculated using a standard curve obtained with dilutions of purified RAGE and data were analyzed by linear regression analysis (correlation coefficient > 99%).* C: *Extracts of cultured cells (RBC), red cell; EC, aortic endothelial cell; VSMC, vascular smooth muscle cell; MC, mesangial cell; MP, mononuclear phagocyte; PC12 pheocbromocytes) were analyzed for RAGE content by ELISA, and data is reported as described above.*

gial cells, mononuclear phagocytes, and PC12 cells demonstrated the presence of RAGE, whereas none was detected in erythrocytes (Figure 8C).

Northern analysis of RNA derived from human organs showed a single band with highest levels of message present in the lung, although lower levels were observed in multiple other tissues (Figure 9A). To be certain that there were not alternate forms of RAGE mRNA, possibly related by alternative splicing, blots were developed for varying times and studies were performed under multiple stringencies. Still, only a single band was apparent as shown in Figure 9A. Based on our immunohistochemical results, we expected that RAGE mRNA would be more evenly and diffusely distributed. To assess expression of the mRNA at the cellular level, *in situ* hybridization was performed. RAGE mRNA was observed in tissue sections present in motor neurons (Figure 9B, sections probed with the sense control were unstained, C), smooth muscle cells (Figure 9D, sections probed with the sense control were unstained, E), and in endothelium (Figure 9F). In other sections, staining for RAGE mRNA was observed in cardiac myocytes and tissue mononuclear phagocytes (data not shown). Similarly, RAGE mRNA was visualized in cultured smooth muscle cells (Figure

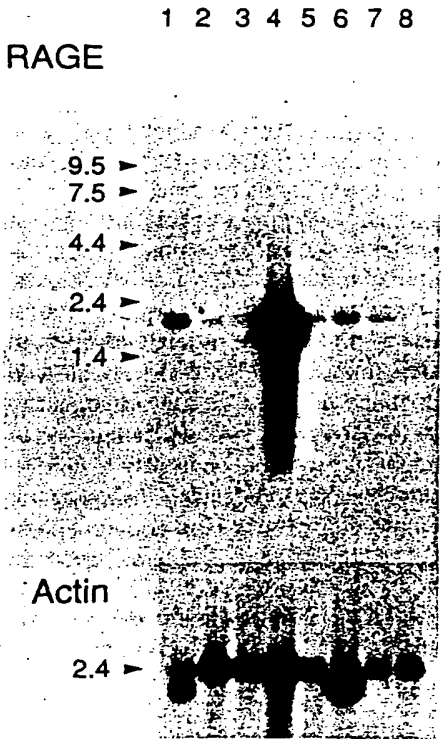
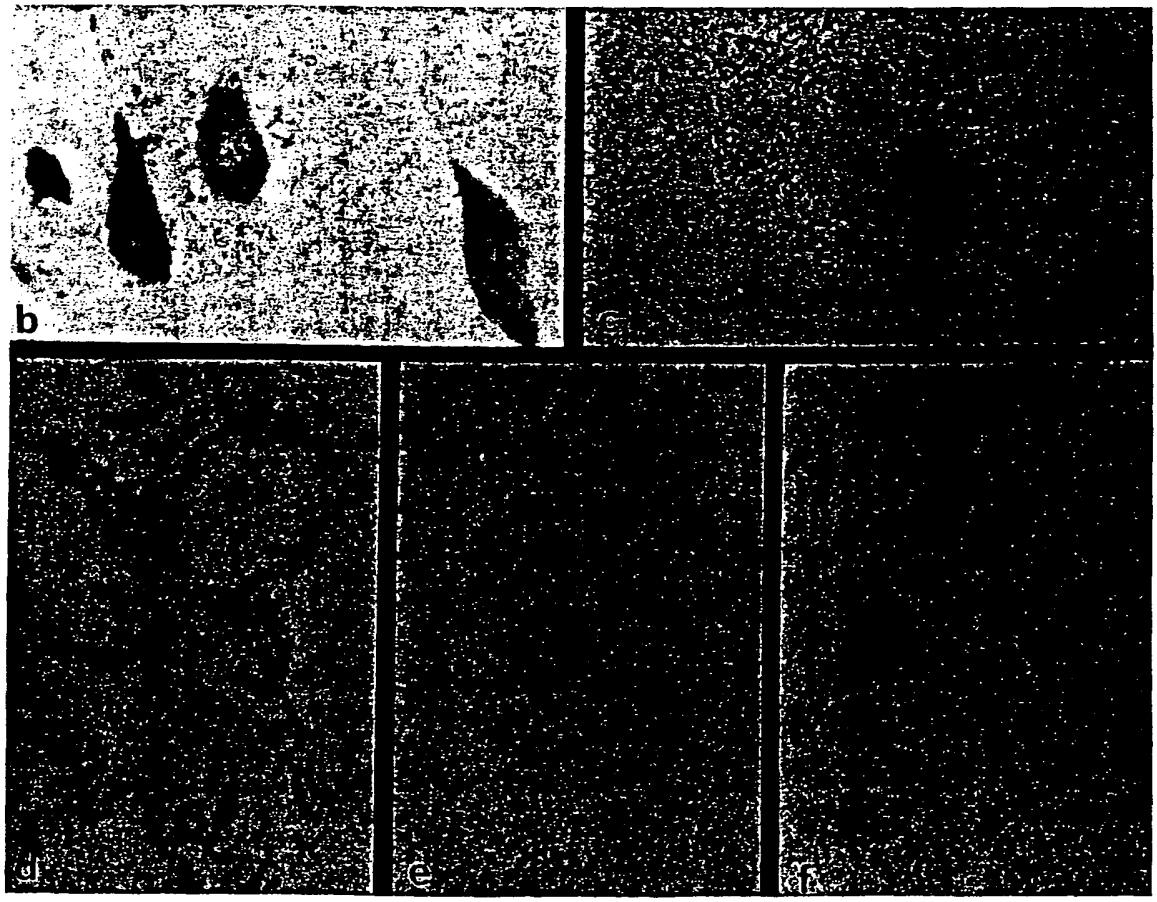


Figure 9. *Northern blot analysis and in situ hybridization for RAGE mRNA.* A: *Northern blotting of human tissue RNA for RAGE mRNA. Two micrograms of human poly A⁺ RNA per lane were loaded, gel separated, and transferred as indicated in the text. The tissue RNA sources are listed in order left to right from lanes 1 to 8: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. RNA molecular weight markers are indicated on the left in kilonucleotide bases. Shown below is the same blot after washing and reprobing with the human actin gene.* B *to* F: *In situ hybridization study of RAGE mRNA expression in bovine tissues: spinal cord with antisense probe* (B), *spinal cord with sense probe* (C), *pulmonary vessel with antisense probe* (D), *pulmonary vessel with sense probe* (E), *and endothelium of a spinal cord blood vessel with the antisense probe* (F). *Note that probing of sections identical to that in* F *with the sense probe resulted in no staining (data not shown). Magnification:* ×*300* (B *to* F).



10A, cultures stained with the sense probe were unstained, B), in adrenal capillary endothelial cells (Figure 10C), mesangial cells (Figure 10D), and mononuclear phagocytes (Figure 10E). Thus, RAGE mRNA is expressed in multiple cell types, though at different levels.

Discussion

Previous studies have defined RAGE in molecular terms, based on biochemical characterization and cDNA cloning[5,6], as well as its presence on cultured endothelial cells and mononuclear phagocytes.[5,7] The 293 cells transfected with RAGE cDNA expressed the antigen on the cell surface and on Western blotting demonstrated a major band with M_r approximately 50 kd, as well as minor bands with M_rs from below 30 to 55 kd.[6] This range of apparent molecular masses of RAGE is consistent with that observed on Western blotting of tissue extracts, where forms from 23 to 48 kd were observed. Although these bands might arise from nonspecific proteolysis during preparation of the tissue extracts, note that in each organ studied RAGE migrates as a characteristic set of two distinct bands. This pattern of presumed posttranslational processing was constant in the same organ derived from different species (mouse, rat, human). Especially in view of the absence of evidence for heterogeneity of RAGE due to alternative splicing at the mRNA level, these data suggest the possibility of tissue-specific, posttranslational processing. Evidence for such processing is supported by the apparent presence of RAGE in intracellular structures, such as Nissl bodies.

Of note is that RAGE mRNA levels detected by Northern blotting do not appear to correlate closely with levels of RAGE antigen. Thus, although the

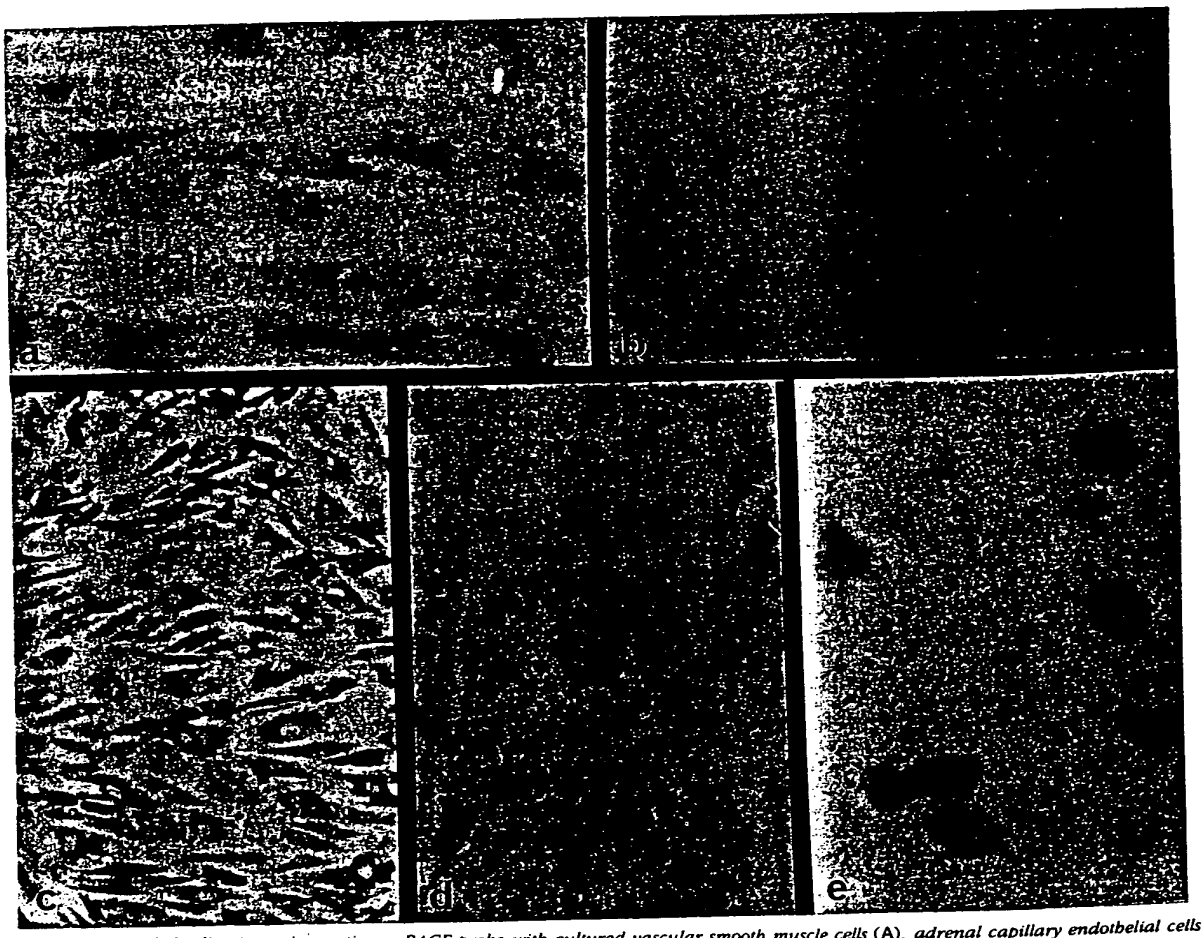


Figure 10. *In situ hybridization using antisense RAGE probe with cultured vascular smooth muscle cells* (A), *adrenal capillary endothelial cells* (C), *mesangial cells* (D), *and mononuclear phagocytes* (E). *In B smooth muscle cells were hybridized with the sense RAGE probe. Control experiments with adrenal capillary endothelial cells, mesangial cells, and mononuclear phagocytes and the sense probe showed no staining, as in* B *with smooth muscle cells (data not shown). Magnification:* ×*300,* (A), ×*120* (B), ×*120* (C), ×*300* (D), ×*600* (E).

lung appears to have much higher levels of mRNA than the heart, RAGE antigen levels are closer than might be expected if the concentration of mRNA were the sole determinant driving expression of RAGE. This suggests that there are likely to be multiple factors regulating translation, posttranslational processing, and degradation of RAGE. Lack of a close correlation between mRNA and antigen levels of proteins has been observed in other situations as well, such as C5a-mediated induction of mononuclear cell interleukin-1 and tumor necrosis factor mRNA. In the latter case, Schindler et al[19] reported comparable levels of cytokine message induction with C5a to that observed after exposure of mononuclear cells to lipopolysaccharide. However, C5a resulted in 12 times less interleukin-1 and 70 times less tumor necrosis factor protein production than was observed in mononuclear cell cultures stimulated with endotoxin, even though message stability was unchanged.[19]

RAGE purified from bovine lung had M_r approximately 30 to 35 kd, which has been considered to be the NH_2-terminal two-thirds of the molecule, based on sequence analysis.[5,6] Our purification procedure for RAGE from lung selectively isolated the 30 to 35-kd form, although a band with this M_r was difficult to discern on Western blots of unconcentrated crude lung extract. This could be due to proteolysis occurring during the purification procedure and/or preferential interaction of the approximately 30 to 35-kd form with the chromatographic resins used. Based on immunological criteria (Figures 1, 2 and 8), these forms of RAGE appear to be indistinguishable when studied with the two different polyclonal antibodies used in these studies, one raised against bovine lung RAGE and the other against recombinant human RAGE. Furthermore, the apparent heterogeneity of RAGE could account for bands with M_r's of 30, 40, and 50 kd observed by Skolnik et al[20] based on ligand blotting using ^{125}I-AGE albumin and extracts of cultured rat mesangial cells, and the 60-kd band purified from rat liver by affinity chromatography with immobilized AGE by Yang et al.[8] However, future studies to further define these molecules[8] will be required to clarify this issue, because the reported NH_2-terminal sequence of the 60-kd liver-derived AGE binding protein is distinct from that of RAGE.

RAGE in normal bovine tissues is expressed in cells of the vessel wall, both endothelial and smooth muscle cells. The antigen appears to be diffusely distributed in the endothelium of large and small vessels in the arterial, venous, and capillary beds. Although the function of RAGE in the vessel wall is not clear, studies in tissue culture have suggested AGEs can modulate a range of endothelial properties including accelerated growth, enhanced thrombogenicity, and increased permeability.[21,22] The presence of RAGE in neural and cardiac tissue raises the possibility that AGE-modified proteins may selectively interact with and modulate properties of these cells as well. For example, pilot experiments with PC12 cells suggest that RAGE is functional, and AGE ligands can mediate induction of cellular oxidant stress, which is blocked by anti-RAGE IgG (data not shown). In this context, given that RAGE, expressed in diverse cell types, is a new member of the immunoglobulin superfamily, it is possible that AGEs may be accidental ligands for RAGE, and in homeostasis the receptor might interact with a yet to be identified ligand, potentially one having a physiological role.

Regulation of RAGE expression will be an important issue to explore in pathological settings. For example, PC12 cells in culture express RAGE, whereas only occasional adrenal chromaffin cells stain for RAGE, suggesting a low level or heterogeneous expression in the quiescent state *in situ*. In this context, the mononuclear cells infiltrating AGE-adsorbed PTFE mesh and in the intima of the atherosclerotic plaque stain intensely for RAGE compared with Kupffer cells or mesangial cells in the normal liver and kidney, respectively. The apparently enhanced staining of RAGE in cultured mesangial cells compared with those in the normal kidney also suggests the possible relevance of factors regulating RAGE expression.

These data provide a foundation for future studies examining RAGE expression in a spectrum of organs and pathological conditions. In view of the presence of RAGE in tissues, a central question concerns whether AGE-RAGE interaction mediates important functional and structural changes contributing to the development of complications in diabetes and other disorders. Understanding the properties of RAGE and the preparation of reagents that selectively block the receptor will be essential for defining its role in pathological settings.

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